

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: TADA et al.

Application No.: 10/537,320

Filing Date: June 2, 2005

For: DENDRITE ELONGATION INHIBITOR FOR
MELANOCYTE AND SKIN PREPARATION FOR EXTERNAL
USE CONTAINING THE SAME

Art Unit: 1612

Examiner:
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DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O.Box 1450
Alexandria, VA 22313-1450
Dear Sir:

I declare as follows:

1. I am employee of Pola Chemical Industries Inc. located at 6-48, Yayoi-cho, Suruga-ku, Shizuoka-shi, Shizuoka, Japan, which is engaged in the business of production and sale of cosmetics.
2. I am one of co-inventors of the above-identified patent application.
3. The actions of Centaureidin and Nobiletin (EP 1147764) to melanocyte are clearly different from each other. Nobiletin inhibits production of melanin, however, Centaureidin does not inhibit production of melanin.
4. I have conducted the experiments described herein and present them as evidence supporting the above fact.
5. Test Example: Test on Inhibition of Melanin Production using Cultured Normal Human Melanocyte
Inhibitory actions of Centaureidin and Nobiletin on melanin production

was evaluated by using 2-thiouracil (^{14}C -labelled 2-thiouracil in this test) specifically incorporated into melanin in the process of intracellular synthesis of melanin.

A 24-well microplate was used and 2 ml of complete medium for culturing melanocytes (product of KURABO INDUSTRIES LTD.) was added to each of 21 wells of the plate. Into each of the 21 wells, normal human melanocytes (product of KURABO INDUSTRIES LTD.) were inoculated at a concentration of 1.5×10^4 cells/cm² and cultured at 37°C in 5% carbon dioxide atmosphere for 24 hours.

Subsequently, the media of all of the above 21 wells were replaced as follows:

- in respect of three wells, the medium was replaced by new complete medium for culturing melanocytes (control wells);
- in respect of nine wells, the medium was replaced by complete medium for culturing melanocytes containing Centaureidin at concentrations of 0.1 μM , 0.5 μM and 1 μM each for three wells; and
- in respect of another nine wells, the medium was replaced by complete medium for culturing melanocytes containing Nobiletin at concentrations of 1 μM , 5 μM and 10 μM each for three wells.

Further, 2-[2- ^{14}C]thiouracil (^{14}C -labelled thiouracil) was added at $0.25 \times 10^{-6}\text{Ci}$ (curie). The cells were further cultured under the same conditions as above for three days. After the cultivation was completed, the culture broth was removed from each of the wells, and each of the wells was washed with PBS (phosphate buffered saline). Thereafter, cells were peeled off from the bottom surfaces of wells by using a medium containing trypsin and EDTA to thereby prepare a cell-suspension liquid, and cells were collected from the liquid by centrifugation. The number of the cells was counted by using a hemocytometer. Subsequently, the amount of 2-[2- ^{14}C]thiouracil ($0.25 \times 10^{-6}\text{Ci}$) contained in the cells collected from each of the wells was measured by using a liquid scintillation counter (product of ALOKA CO., LTD.). The percentage of the radiation dose detected from the cells cultured in each of the media containing a substance to be evaluated against the radiation dose detected from the cells collected from the control wells was calculated and defined as melanin amount(%). That is, it can be assumed that the smaller the radiation dose incorporated in each cell is, the smaller the melanin amount is, and thus the higher the inhibitory action of the added substance is.

6. Results

The results are shown in the following table.

Nobiletin inhibits production of melanin, however, Centaureidin does not inhibit production of melanin.

Compound (concentration)	Melanin amount (%)
Centaureidin (0.1 μ M)	101.36 \pm 3.57
Centaureidin (0.5 μ M)	102.68 \pm 6.52
Centaureidin (1 μ M)	100.86 \pm 5.41
Nobiletin (1 μ M)	83.67 \pm 5.77
Nobiletin (5 μ M)	70.03 \pm 5.06
Nobiletin (10 μ M)	53.62 \pm 6.35

7. I further declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: 
Akihiro TADA

Date: June 24 , 2010